Original Paper

Immunoexpression of N-cadherin, Twist and Vimentin in Bladder Urothelial Carcinomas

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ABSTRACT: Purpose: Epithelial mesenchymal transition consists in the acquisition of neoplastic epithelial cells of a mesenchymal phenotype the process being involved in cancers invasion and metastasis. In this study were analyzed the expression of N-cadherin, Twist and Vimentin in bladder urothelial carcinomas according to the main prognostic parameters. **Material/Methods**: The study included 20 bladder urothelial carcinomas which were analyzed histopathological, immunohistochemical and statistical. **Results**: N-cadherin was identified in 45% of cases, which belonged to high-grade carcinomas with deep invasion and lymph node metastases. Twist immunoreaction was identified in all cases and was significantly increased in advanced stages carcinomas. Vimentin was present only in the advancing edge in 25% of cases, which belonged to highly invasive carcinomas. Urothelial carcinoma metastases were N-cadherin and Twist and Vimentin negative. We found a linear positive distribution of N-cadherin and Twist values. **Conclusion**: the used markers are useful for identifying aggressive urothelial carcinomas in the context of reciprocal stimulation mechanisms inside of urothelial epithelial-mesenchymal transition process.

KEYWORDS: *urothelial carcinoma, epithelial-mesenchymal transition*

Introduction

Bladder cancer is on the 7th place among malignant neoplasms worldwide, being responsible for about 150,000 deaths annually [1,2]. Although over 75% of cases are diagnosed at an early stage, over 50% relapse within the first year [3,4]. Also, cases with invasion into the muscularis propria have a poor prognosis, with a 5-year survival between 15-68% [5]. Variability of relapse and survival rate of patients emphasizes the importance of knowledge of biomolecular mechanisms involved in urothelial carcinogenesis.

Epithelial mesenchymal transition (EMT) is a process in which epithelial cells acquire a mesenchymal phenotype [6-9]. EMT is a process involved in embryogenesis (type I), healing processes (type II) and oncogenesis (type III) [10]. The process of de-differentiation mediated by EMT is currently accepted as a key process in tumor progression, being involved in cancers invasion and metastasis [6,11].

EMT process can be evaluated through mesenchymal (vimentin, matrix-metaloproteinases- MMP, α- smooth- muscle actin) or epithelial markers (cytokeratin, E-cadherin, N-cadherin) or by transcription factors and some growth factors (Smad, Snail, β-catenin, Twist, FGFR) [7-9]. The cadherins switch, in which E-cadherin expression is lost and N-cadherin (mesenchymal cadherin) is overexpressed and basically replaces E-cadherin, increasing level of transcription factors and expression of mesenchymal markers in tumor epithelial cells are studied events in different locations of carcinomas [8,12]. Nevertheless, in the case of urinary tract malignancies, including bladder some authors indicate weak representation of studies, although investigations in this context can provide efficient therapeutic targets [6,13].

In this study were analyzed the expression of N-cadherin, Twist and Vimentin in bladder urothelial carcinomas according to the main prognostic parameters.

Material and methods

In this study we analyzed 20 bladder urothelial carcinomas from patients hospitalized in Urology Clinic of Emergency County Hospital of Craiova which receiving cystectomy. Pathological diagnosis was made in the Laboratory of Pathology of the same hospital in the period 2014-2015. The specimens were fixed in 10% buffered neutral formalin, processed for paraffin embedding and Hematoxylin–Eosin staining. As morphological parameters we investigated degree of differentiation, depth of invasion, lymph node status, pTNM stage. For the assessment of the lesions we used the WHO 2004 staging system [14]. In this study included cases of papillary carcinoma of the urothelium, who had not received prior oncological therapy

and without distant metastases (M0). The study was approved by the local ethical committee, and written informed consent was obtained from all the patients. For immunohistochemical analysis we used a panel of antibodies, as shown in the table below (Table 1).

Table 1. The antibodies panel

Antibody	Clone	Dilution	Antigen retrieval	External positive control
N-cadherin	6G11/ Dako	1/30	HIER, Citrate, pH 6	tonsil
Twist	Twist 1 /LSBio	1/1000	HIER, Citrate, pH 6	tonsil
Vimentin	SP20/ Thermo Scientific	1/50	HIER, Citrate, pH 6	prostate

Immunohistochemical reactions were performed on serial sections. After antigen retrieval, endogenous enzyme blocking, and unspecific sites' blocking, the sections were incubated overnight at 4°C with the Twist, N-cadherin and Vimentin monoclonal antibodies. The next day, the sections were incubated with biotinylated secondary antibodies, which were later on amplified with the LSAB 2 HRP system (DAKO, Redox, Bucharest, Romania, code K0675) and visualized with 3,3`, diaminobenzidine tetrahydrochloride (DAB, Dako, code 3467). Finally, the slides were counterstained with hematoxylin and coversliped with DPX (Fluka, Redox).

To quantify the results of immunohistochemical reactions, we used indexes of positivity (IP), by reporting the number of labeled cells to total cells at 20x microscopic field (MF). For each case were analyzed 10 MF. Also, reactions were analyzed in relation to tumor topography (intratumoral versus advancing edge), We used external positive controls (Table 1) and respectively negative controls, by omitting the primary antibody. For quantification in the case of Twist and Vimentin epithelial tumor areas were selected. The acquisition of the images was done on a Nikon Eclipse E600 microscope and with the software package Lucia 5. For the statistical analysis was create an electronic database and the results were compared using Student t-test (SPSS, Inc., Chicago, IL, USA). Also, we used Pearson test and One-way ANOVA test to assess the differences between more than two independent groups. All central tendencies were reported as average ± standard deviation (SD). Results were considered significant for p values <0.05.

Results

In this study prevailed high grade urothelial carcinomas (55%), with invasion in the muscularis propria (pT2- 40%) without metastases in regional lymph nodes (85%) and tumor stage II (40%) - Table 2.

Table 2. Cases distribution depending on the investigated parameters.

Parameter	Variable (No)
Differentiation degree	LG= 9 HG= 11
Depth of invasion	T1= 5 T2= 8 T3= 7
Lymph node metastasis	N0= 17 N1= 3
Stage	I= 5 II= 8 III= 4 IV= 3

Note: LG (low grade); HG (high grade)

N-cadherin membrane and cytoplasmic immunoreaction has been identified in nine examined cases (45%). Positive carcinomas present high grade, with muscularis propria / whole bladder wall invasion and lymph node metastases and found no significant differences between intratumoral and advancing edge compartments. N-cadherin positivity index (IP) ranged from 25-65, with a mean of 38.8 ± 17.8/MF (fig.1a-b). Reaction of N-cadherin was present in one case of low-grade urothelial carcinoma with invasion into the muscularis propria and IP <10% (fig.1c-d).

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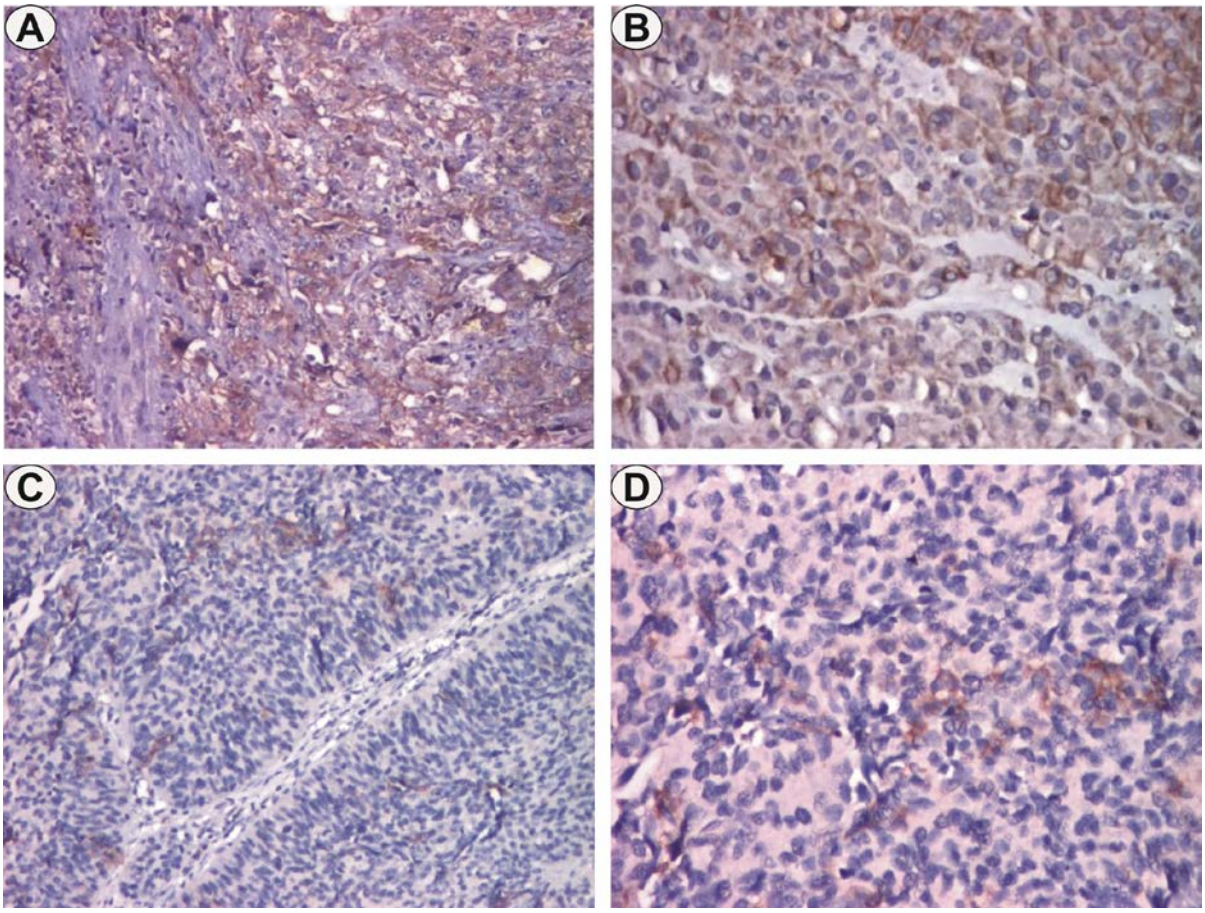

Fig.1. N-cadherin immunostaining. A) High grade urothelial carcinoma, advancing edge x100; B) High grade urothelial carcinoma, intratumoral, x200; c) Low grade urothelial carcinoma, x100; c) Low grade urothelial carcinoma, x200

In relation to the depth of invasion we found higher IP mean values of invasive throughout the entire wall carcinomas (T3, IP = 48 ± 13.5 / MF) compared with those in muscularis propria (T2, IP = 27.5 ± 17 / MF) and this was not statistically significant (p> 0.05, Student's t-test). Also we found no significant differences in the expression of N-cadherin in relation to tumor stage and lymph node metastases (p> 0.05, Student's t-test).

Twist nuclear immunoreaction was present in all analyzed cases. The reaction was identified in tumor cells as well as in the stromal elements as endothelial cells, fibroblasts, lymphocytes, plasma cells. Intratumoral and advancing edge IP Twist medium values were 61.4±10.8/MF and respectively 59.5±9.58/MF which was no statistically significant (p>0,05, Student's t-test). Although Twist expression analysis in relation to the degree of tumor differentiation indicated higher values of IP in high-grade carcinomas compared to low grade both intratumoral (65.4 ± 10.5 / 54.7 ± 10.3 vs MF / MF) and at advancing edge (62.2 ± 10.8 / 53.8 ± 9.9 vs MF / MF), we found no statistically significant differences (p> 0.05, Student's t-test) (fig. 2a-d).

In relation to the depth of invasion, invasive carcinomas in whole bladder wall (T3) had a intratumoral IP Twist of 71.1 ± 8.2 / MF, respectively of 65.0 ± 10.8 / MF at the advancing edge. By comparison, muscularis propria invasive carcinomas (T2) presented intratumoral IP Twist of 59.3 ± 8.6 / MF, and 56.8 ± 9.6 / MF in the advancing edge, and for lamina propria invasive carcinomas the were 51 ± 4.1 / MF, respectively 48 ± 7.5 / MF. The statistical analysis indicated significant differences in relation to the depth of invasion, both intratumorally (p = 0.001, ANOVA test) and at the advancing edge (p = 0.026, ANOVA test) (fig. 32a-b). The same aspect was observed in the analysis of Twist expression in relation to the tumor stage, the tumor carcinomas in stage I-II presented mean values of Twist IP intratumoral (71.1 ± 8.82 / MF) and at the advancing edge (65 ± 9.3 / MF) higher than those in stages I-II (56.1 ± 8.2 / MF, respectively 53.4 ± 9.6 / MF), Anova test indicating significant differences (p = 0.004, p = 0.043) (fig. 3c-d).

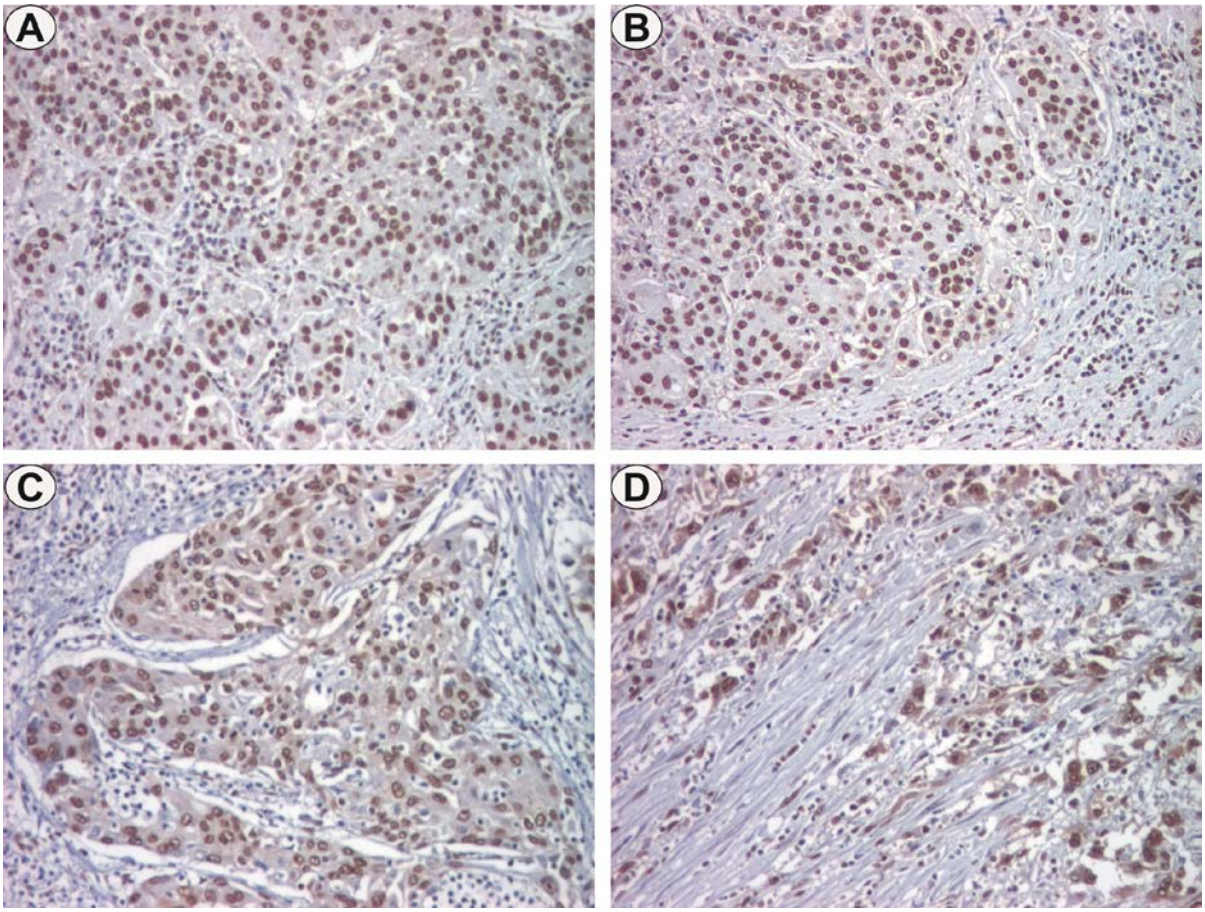

Fig.2. Urothelial carcinomas, Twist immunostaining, x100. A) Low grade, intratumoral; B) Low grade, advancing edge; C) High grade, intratumoral; D) High grade, advancing edge

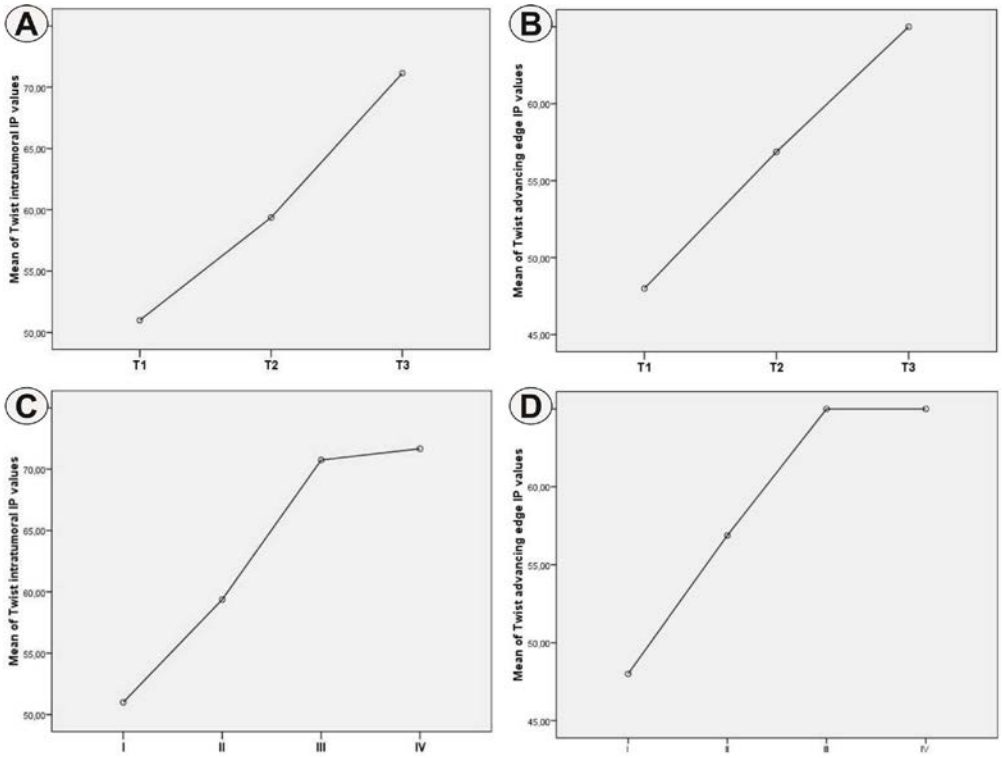

Fig.3. A) Intratumoral IP Twist medium values depending on deepth of invasion (T); B) Advancing edge IP Twist medium values depending on deepth of invasion (T); C) Intratumoral IP Twist medium values depending on tumor stage; B) Advancing edge IP Twist medium values depending on tumor stage

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Although Twist IP values were higher in all compartments urothelial carcinomas tumor that presenting metastases to regional lymph the aspect was not statistically significant (p> 0.05, Student's t-test).

Vimentin cytoplasmic imunoexpression in the tumor cells was found in five cases (25%). Urothelial carcinoma that presented Vimentin positive reaction were high grade with deep invasion into the muscularis propria (T2) or in the entire bladder wall (T3), the reaction being identified only in the tumor advancing edge and medium Vimentin IP value was 25.2 ± 4.3 / MF (fig.4a-b). We found no statistically significant differences in the expression of vimentin in relation to the depth of invasion, tumor stage or lymph node metastases (p> 0.05, ANOVA or Student's t-test).

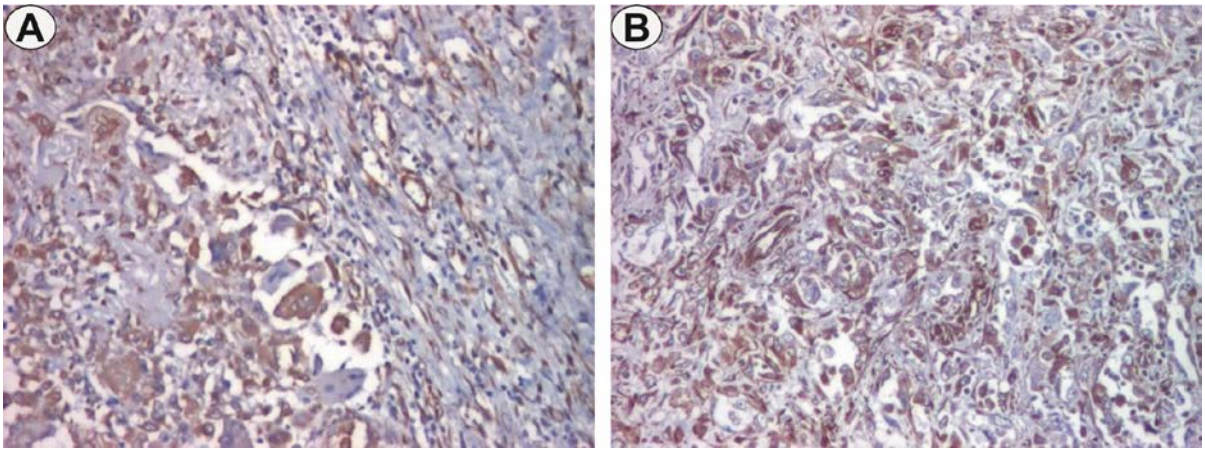

Fig.4. A) High grade urothelial carcinomas, Vimentin immunostaining, lamina propria invasion, x100; B) High grade urothelial carcinomas, Vimentin immunostaining, muscularis propria invasion, x100

The analysis of IP values for the analyzed markers indicated a positive linear distribution of N-cadherin and Twist in advancing edge but it was not statistically significant (p> 0.05, Pearson test).

In the lymph node metastases, vimentin expression was absent. All lymph node metastases from urothelial carcinoma were N-cadherin and Twist positive, with focal markings fo N-caderine and diffuse for Twist, without significant differences when compared to primitive tumors (fig.5a-b).

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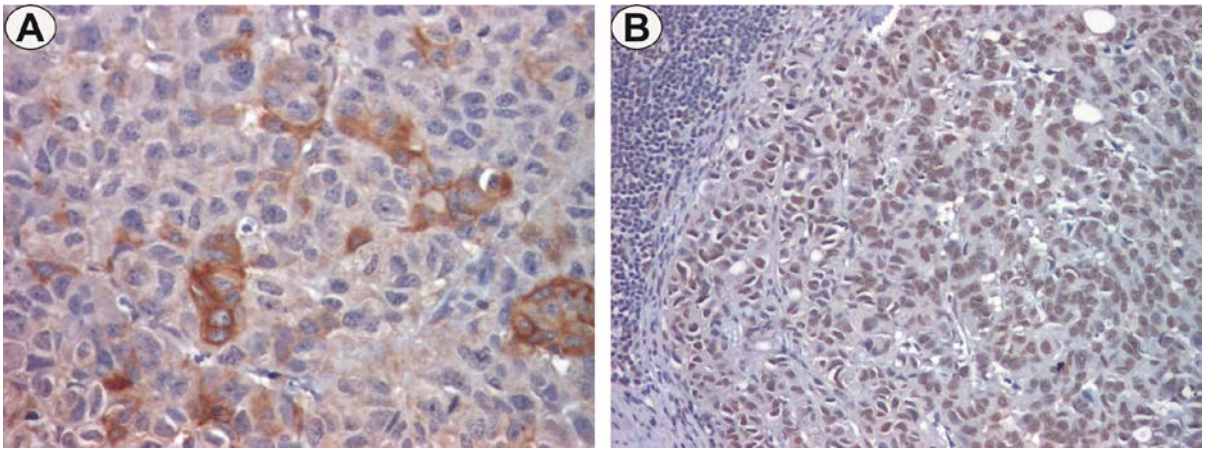

Fig.5. A) Urothelial carcinoma, lymph node metastasis, N-cadherin immunostaining, x200; B) Urothelial carcinoma, lymph node metastasis, Twist immunostaining, x100;

Discussions

EMT is considered a latent embryonic biomolecular program, which is activated in malignant tumors, cells exhibiting a migratory, invasive and metastatic phenotype [15,16].

The cadherins swich respectively E-caderinei - N-cadherin replacement, has been indicated in numerous studies on carcinomas, including those of the urinary bladder [8,17-19]. In our study the expression of N-cadherin was identified in 45% of analyzed carcinomas, with high grade and muscularis propria / entire bladder wall invasion and lymph node metastases, without significant differences between intratumoral compartments and the advancing edge. Literature data indicate various aspects of N-cadherin expression related to bladder tumors. In a study conducted on a group of 51 urothelial carcinomas, Rieger-Christ KM et al. investigated the expression of cadherins and found that N-cadherin was not expressed in normal urothelium and was observed in 39% of analyzed carcinomas, most of them being invasive [17]. Other authors have indicated the absence of N-cadherin in the normal urothelium Ta carcinoma, the positivity in over 80% of the T1 carcinomas, being expressed in more than 60% of T2-T4 carcinomas [1,20]. In a study conducted in 2007, Baumgart E et al. examined the expression of several markers involved in urothelial EMT and indicated N-cadherin as only one did not correlate with tumor stage and grade [18]. The aspect was described in 2006 after Lascombe I et al. indicated the absence of N-cadherin in pTa carcinomas, being significantly decreased in pT1-T2 compared to T3 carcinomas, but without relation to tumor stage and grade [21]. Moreover, in 2010 Jäger T et al., analyzing the N-cadherin gene expression in urothelial carcinoma indicated that muscularis propria invasive and N-cadherin negative (40%) carcinomas had a poorly prognosis compared to the immunopositive cases, aspect that was attributed to alternative pathways of N-cadherin action in carcinoma progression [22]. In this context the variable expression of N-cadherin appears to be consistent with urothelial carcinoma heterogeneity.

As regulator of intercellular adhesion and cell migration, Twist, along with other transcription factors as Slug, Snail, Zeb, appear to play key roles in urothelial EMT [8,23]. In this study Twist expression was found in all cases, with significant differences in relation to the depth of invasion and tumor stage, invasive and advanced stages carcinomas presenting upper immunostains. We have not found differences in Twist expression in metastases compared with primitive carcinomas. In a study from 2007, Zhang Z et al. indicated in urothelial bladder carcinomas Twist overexpression compared to normal urothelium and association of Twist expression with high degree and advanced tumor stage [24]. The same authors indicated increased Twist expression in metastases compared to the primitive tumor [24]. Also in other studies Twist expression was associated with poor prognosis, high degree and advanced stage, with overexpression in tumor metastases compared to the primitive tumors [25,26].

Investigation of Twist expression in urothelial carcinomas is an attractive target especially since there is some evidence that reducing expression of transcription factors leads to restoration of E-cadherin expression and response to conventional therapy [27]. Thus, investigating the expression of Twist, E-cadherin and beta-catenin in bladder carcinomas, Shen CH et al. indicates increasing Twist expression with tumor grade, its inhibition inducing increased expression of E-cadherin and decreased of beta-catenin expression [28].

The acquisition of mesenchymal phenotype by carcinomas is a mechanism investigated in EMT process and which is directly related to tumor invasiveness [11]. Data from the literature indicate that the EMT is located at the advancing edge, where vimentin expression increases and E-cadherin expression is lost [6]. In our study Vimentin expression was found only in 25% of cases at the advancing edge of high-grade carcinomas without relation to depth of invasion, tumor stage or the presence of metastases. Also, the reaction was negative in the analyzed lymph node metastasis.

Literature data indicates increased Vimentin expression in G3 invasive carcinomas compared to the G1 / G2 and is increased in the invasive tumors compared with the superficial ones [13]. The same issue was described by Baumgart E et al. in 2007, indicating the association of vimentin expression with in invasive high-grade urothelial carcinomas [18]. Thus, in over 30% of invasive carcinomas vimentin appear to be overexpressed in the tumor cells [26].

In our study we found a positive linear relationship of N-cadherin and Twist indicating stimulatory mechanisms between the two proteins. Literature data indicate that Twist is involved in the activation of N-cadherin and the E-cadherin inactivation [16]. Furthermore, there

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are links between transcription factors: thus Snail increase stability of Twist, which in turn activates transcription of Slug [29]. It also transcription factors activates proinvazive gene that regulate vimentin, fibronectin, matrix-metalloproteinases expression [16]. For understanding the connections of biomolecular markers involved in EMT as N-cadherin, Twist and Vimentin further studies are needed on large lots, essential for selecting therapeutic targets and carcinomas for which these targets can be effective.

Conclusions

In this study, N-cadherin and Vimentin were expressed in high-grade carcinomas with depth invasion. Vimentin was present only in the advancing edge. The aspect indicates the acquisition of a mesenchymal phenotype of aggressive urothelial carcinomas. Twist expression was present in all cases, being significantly higher in carcinomas in advanced stages, that indicates the involvement of the transcription factor in different stages of tumor progression. The markers used in this study appear to be useful for identifying aggressive urothelial carcinomas. Linear positive distribution of N-cadherin and Twist values indicate possible reciprocal stimulatory mechanisms within urothelial epithelial-mesenchymal transition process.

Acknowledgments

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